The Action of Digitonin on Rat Liver Mitochondria

THE EFFECTS ON ENZYME CONTENT

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1. The effects of repetitive treatment of rat liver mitochondria with digitonin were examined. The first treatment results in the removal of the outer membrane. Almost all the NADH-cytochrome *c* reductase (rotenone-insensitive) is lost whereas the major portions of the soluble and bound enzymes are retained. One exception appears to be the cytochromes, which undergo somewhat larger losses. The resulting inner-membrane complex carries out oxidative phosphorylation and P_1 -ATP exchange. 2. The properties of the inner-membrane complex are affected by the osmoticity of the medium. When it is suspended in water little protein is lost but there is a marked loss of phosphorylation. If after the suspension in water the particulate fraction is reisolated by centrifugation and treated with digitonin, or if the aqueous suspension is treated directly with digitonin and the particulate fraction then reisolated, the phosphorylation is largely restored. 3. Additional treatment of the inner mitochondrial complex with digitonin results in the formation of a particulate fraction that contains approx. 8% of the initial mitochondrial protein, no outer membrane, no soluble mitochondrial enzymes and is still capable of coupled oxidative phosphorylation and P_1 -ATP exchange. These effects cannot be reproduced by treatment with water. 4. The rat liver mitochondria and all of the resulting preparations obtained after digitonin treatment may be stored for long periods in dimethyl sulphoxide with little change of activity.

One of the earliest methods devised for studying oxidative phosphorylation in disrupted mitochondria involved treatment with digitonin (Cooper & Lehninger, 1956). A considerable amount of information has been obtained with this preparation, but in some studies apparent discrepancies have appeared. Cooper & Kulka (1961) and Kulka & Cooper (1962) reported that the ADP-ATP exchange activity was stimulated by phosphate and inhibited by azide and sucrose, whereas Wadkins & Lehninger (1958) reported no effects with these compounds. These differences have been attributed to variations in the degree of mitochondrial disruption produced by digitonin with the somewhat different preparative conditions employed (Wadkins & Lehninger, 1963). This suggestion raises the question of the origin and nature of the mitochondrial fragments isolated after digitonin treatment. The earlier digitonin preparations were free of many mitochondrial enzymes and were isolated by differential centrifugation between 50 000 and 100 000*g*, suggesting that they consisted of relatively small particles. They are now known to be heavily contaminated with outer membrane (see the

Discussion section). Since they carry out oxidative phosphorylation it has been assumed they arise at least in part from the inner membrane. However, the phosphorylation activity may reside in intact inner-membrane 'ghosts', pieces of disrupted inner membrane or particulate material originating in the mitochondrial matrix. The existence of the latter has been suggested by Whittaker (1963). The nature of the effects produced by digitonin is also poorly understood. They may reside in a specific action on the stability of the outer or inner membranes or perhaps both. They may also arise by a combination of membrane labilization with the effect of the hypo-osmotic conditions on the structures resulting from the prior action of digitonin.

We have undertaken a more complete analysis of the action of digitonin and have examined the effects of repeated exposure of mitochondria to this agent on (1) the distribution of some selected enzymes, (2) the structures resulting from these treatments as evaluated by electron-microscopic examination and (3) the lipid composition of each resulting structure.

While this work was in progress Levy, Toury & Andre (1966) and Schnaitman, Erwin & Greenawalt (1967) have reported that digitonin appears to disrupt the outer mitochondrial membrane preferentially. Schnaitman *et al.* (1967) suggested that this selectivity may reside in a marked difference in cholesterol content, the presumed site of action of digitonin, between the outer and inner membranes.

EXPERIMENTAL

Mitochondria. Mitochondria were isolated from the livers of Wistar-strain rats weighing 175–200 g. (Carworth Farms, New City, N.Y., U.S.A.) by differential centrifugation (Sorvall RC-2B centrifuge) of a 17% homogenate in 0.3 M-sucrose and 2 mM-EDTA, pH 6.8. The SS-34 rotor was used throughout with the exceptions noted below. The cellular debris and nuclear material were removed by centrifuging for 15 min. at 425g (GSA rotor). The mitochondria were isolated from the supernatant solution by centrifuging for 15 min. at 12000g (GSA rotor). The mitochondria were then washed twice in 0.3 M-sucrose with one-half and one-quarter the original homogenate volume and reisolated by centrifuging for 10 min. at 14500g. They were finally resuspended to a protein concentration of 130–160 mg./ml. of 0.3 M-sucrose. The yield of mitochondria was 1.9 ± 0.3 g. of mitochondrial protein/100 g. wet wt. of liver (20 experiments).

Preparation A. A solution of 10% (w/v) digitonin in water was added to a suspension of mitochondria to give a final concentration of digitonin of 1% and a ratio 1:1 mg. of digitonin/10 mg. of mitochondrial protein. The concentration of sucrose was 0.26–0.27 M. This suspension was incubated for 20 min. at 0° with constant mixing, diluted with 3 vol. of 0.3 M-sucrose and centrifuged at 12000g for 10 min. A tan-coloured pellet (A) with a thin brown fluffy layer and a yellowish-brown supernatant fraction resulted. The fine brown fluffy layer was decanted with the supernatant fraction and the tan pellet was washed twice in 0.3 M-sucrose with one-half the volume of the diluted digitonin suspension. A suspension of the washed pellet containing 3.6 mg. of protein/ml. of 0.3 M-sucrose was used for the experiments shown in Table 5. The supernatant solution from the 12000g centrifugation plus the washes constituted the crude outer-membrane fraction that was used to determine enzyme distributions and recoveries shown in Tables 1–4.

Preparation B. Preparation A was resuspended in 0.01 M - *N* - tris(hydroxymethyl)methyl - 2 aminoethane - sulphonic acid (adjusted to pH 6.8 with NaOH) to approx. 130–160 mg. of protein/ml. and 10% digitonin (w/v) added to give a final concentration of digitonin of 1% and a ratio 1:1 mg. of digitonin/10 mg. of protein of preparation A. The suspension was incubated for 20 min. at 0° with constant mixing and then diluted with 3 vol. of cold triple-distilled water. A preliminary centrifugation at 12000g for 10 min. resulted in a small tan pellet, which was washed once in water. The pellet contained 4–8% of the protein and consisted of unbroken and partially disrupted preparation A. The pellet was discarded and the supernatant fractions were combined and centrifuged at 45000g for 20 min. The resulting pellet was washed in water and finally resuspended in water (3.6 mg. of protein/ml.).

Preparation C. Preparation B was resuspended in water to approx. 30–50 mg. of protein/ml. and 10% digitonin added to give a ratio 1:1 mg. of digitonin/10 mg. of protein. The final concentration of digitonin was 0.3–0.5%. The mixture was incubated for 20 min. at 0° with mixing, diluted with 3 vol. of water and centrifuged at 45000g for 20 min. The pellet was washed once with water and finally suspended in water (3.6 mg. of protein/ml.).

Storage of preparations. Samples containing 30–40 mg. of protein/ml. of suspending medium were placed in glass tubes or conical flasks and diluted with 0.1 vol. of dimethyl sulphoxide. They were quickly frozen as a thin layer by rapid swirling of the container in ethanol–solid CO₂ mixture and stored at –80°. The samples were thawed by immersing the container in a 22° water bath until thawing just began. The container was then transferred to an ice bath and kept at 0° until thawing was completed.

Enzyme assays. All spectrophotometric assays were carried out at 30° with a Gilford model 2000 instrument. The specific activities were determined from initial rates and were all proportional to enzyme concentration under the conditions described. The amount of protein in the assays varied between 12 μ g. and 240 μ g.

NADH- and NADPH-cytochrome *c* reductases were assayed by a modification of the method of Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967). The reduction of cytochrome *c* was measured at 550 m μ in a 1 ml. system containing 50 μ moles of P_i, 0.1 μ mole of cytochrome *c* and 0.5 μ mole of NaCN (neutralized just before use) for mitochondria and for preparation A and 2 μ moles of NaCN for preparation B. The assay medium for NADPH-cytochrome *c* reductase also contained 3.5 μ moles of rotenone. The final pH was 7.5. The reaction was started by adding 0.1 μ mole of NADH or NADPH.

Malate dehydrogenase was assayed as described by Siegel & England (1961) except that the 1 ml. reaction volume contained 3.5 μ moles of rotenone.

3-Hydroxybutyrate dehydrogenase was assayed as described by Caplan & Greenawalt (1966) except that the 1 ml. reaction volume contained 3.5 μ moles of rotenone instead of Amytal.

Glutamate dehydrogenase was measured by following NADH formation at 340 m μ in a 1 ml. system containing 50 μ moles of P_i, 1 μ mole of NAD⁺ and 3.5 μ moles of rotenone. The reaction was started by adding 30 μ moles of glutamate. The final pH was 7.5.

Fumarase was assayed by the method of Massey (1955) by following the change in extinction at 240 m μ in a 1 ml. system containing 50 μ moles of P_i and 50 μ moles of malate. The final pH was 7.5.

Isocitrate dehydrogenase was assayed according to an unpublished modification by C. Bernofsky (personal communication) of the method of Arrigoni & Singer (1962). The 1 ml. system contained 180 μ moles of tris-HCl buffer [in 1.2% (w/v) bovine serum albumin], 6.7 μ moles of MgSO₄, 2 μ moles of freshly neutralized NaCN, 150 μ moles of trisodium DL-isocitrate, 3.3 μ moles of NADP⁺, 67 μ moles of phenazine ethosulphate (stored in the dark) and 0.1 ml. of 2,6-dichlorophenol-indophenol freshly made so that at pH 7.0 the extinction at 600 m μ is 21. The final pH was 7.8 and the reaction was started by adding enzyme. The extinction of the reaction vessel was approx. 2 and the decrease was measured between 1 and 2.

Succinate dehydrogenase was assayed essentially as

described above for isocitrate dehydrogenase with NADP⁺ omitted, 25 μ moles of succinate as substrate rather than isocitrate and 0.67 μ mole of added phenazine ethosulphate. The reaction was started by adding enzyme.

Adenosine triphosphatase was assayed by measuring the release of P_i by the method of Martin & Doty (1949). The 0.5 ml. system contained 5 μ moles of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, 3 μ moles of ATP and 1.5 μ moles of MgSO₄. The final pH was 7.0. Either 60 μ g. or 120 μ g. of enzyme was added to start the incubation, which was at 23° for 20 min.

Samples for all enzymic assays listed above (1 ml. containing 3.6 mg. of protein) were treated ultrasonically in a Beckman no. 326826 Polyallomer tube for 30 sec. with a Branson Sonifier with 50 w of power. The samples were then used without further modification. These conditions were found to produce maximum activity for all of the enzymes assayed.

Cytochromes *a* + *a*₃, *b*, *c* and *c*₁ were measured according to the method of Williams (1964) with a Cary model 14 recording spectrophotometer. The full-scale extinction was 0–0.2.

Oxidative phosphorylation. All assays were in a total volume of 1.0 ml. that contained 1.0 mg. of bovine serum albumin, 3.8 units of Sigma type 301 hexokinase, 0.6 μ mole of MgSO₄, 10 μ moles of P_i containing approx. 1.5×10^6 counts/min. of ³²P, 4.5 μ moles of ADP and 0.72 mg. of protein. Substrates were 20 μ moles of DL-3-hydroxybutyrate, 0.05 μ mole of cytochrome *c* + 10 μ moles of ascorbate or 10 μ moles of succinate. The final pH was 6.8. Incubations were for 25 min. at 23° with shaking in a water bath. P/O ratios were measured with 3-hydroxybutyrate as substrate. The determinations of acetoacetate formation (Walker, 1954) and P_i uptake (Walters & Cooper, 1965) were made with previously described methods.

P_i-ATP exchange. The following components were present in a total volume of 1.0 ml.: 10 μ moles of P_i containing approx. 1.5×10^6 counts/min. of ³²P, 1.0 mg. of bovine serum albumin, 10 μ moles of ATP and 0.72 mg. of protein. The final pH was 6.8. The incubations were for 25 min. at 23°. The exchange was measured as described by Walters & Cooper (1965).

Chemicals. The phenazine ethosulphate was a gift from Dr Carl Bernofsky. Rotenone (Sigma Chemical Co., St Louis, Mo., U.S.A.) was stored at –20° as a 0.75 mM solution in ethanol. The nucleotides were obtained from P-L Biochemicals Inc. (Milwaukee, Wis., U.S.A.), enzyme-grade sucrose from Mann Research Laboratories (New York, N.Y., U.S.A.), [³²P]P_i from Tracerlab (Waltham, Mass.,

U.S.A.), A-grade digitonin from Calbiochem (Los Angeles, Calif., U.S.A.), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid from General Biochemicals Inc. (Chagrin Falls, Ohio, U.S.A.), cytochrome *c* (type III) from Sigma Chemical Co. and dimethyl sulphoxide from Aldrich Chemicals Inc. (Milwaukee, Wis., U.S.A.). The water used was triple-distilled and the final two distillations were made in a quartz still.

RESULTS

The work described below is an examination of the effects of repetitive treatment of mitochondria with digitonin on the content of some selected enzymes. The first stage results in the removal of the outer mitochondrial membrane. The enzyme distribution between the resulting inner-membrane complex, preparation A, and the crude outer-membrane fraction was examined. Preparation A was then further treated with digitonin to yield preparations B and C consisting of small pieces derived from the inner-membrane complex. The enzyme distributions were also examined at each of these steps.

Before the results obtained are described, it may be useful to recapitulate some pertinent facts. Sottocasa *et al.* (1967) and Parsons, Williams, Thompson, Wilson & Chance (1967) have shown that the outer mitochondrial membrane is similar to microsomes in that both contain NADH-cytochrome *c* reductase activity (rotenone-insensitive). Only the microsomes contain NADPH-cytochrome *c* reductase. Both groups have also indicated that the inner mitochondrial unit resulting from the removal of outer membrane contains very little NADH-cytochrome *c* reductase (rotenone-insensitive). It therefore appears reasonable to assume that the determination of NADPH-cytochrome *c* reductase gives a measure of the presence of microsomes and that the NADH-cytochrome *c* reductase (rotenone-insensitive) does the same for outer mitochondrial membrane.

Microsomal contamination. A single preparation of mitochondria was used for the assays shown in Tables 1–4 and it had a specific activity for

Table 1. *Effect of digitonin treatment on distribution of microsomal and outer-membrane enzymes*

Enzyme	Mitochondria		Recovery (% of total units)			
	Sp. activity (μ mole/min./mg. of protein)	Total units	COM			
			COM	Prep. A	Prep. B	Prep. C
NADPH-cytochrome <i>c</i> reductase	0.0022	5.9	73	24	0.2	0
NADH-cytochrome <i>c</i> reductase (rotenone-insensitive)	0.229	635	95	5	0.4	0
Protein recovery (%)		100	46	52	9	8

COM, Crude outer-membrane preparation.

NADPH-cytochrome *c* reductase of 0.0022 μ mole/min./mg. (Table 1) as contrasted with the literature values for the microsomal fraction of 0.055–0.073 (Phillips & Langdon, 1962), 0.027 (Parsons *et al.* 1967) and 0.044 (Sottocasa *et al.* 1967). In six experiments the values obtained for mitochondria were 0.0056 ± 0.0024 μ mole/min./mg. (all variations are presented as means $\pm$ s.d.).

Removal of outer membrane. The first step in the fractionation of the mitochondria after treatment with digitonin involved the isolation of a pellet consisting of inner-membrane structures resulting from the removal of the outer membrane. The ratio of digitonin to protein is crucial at this step, as too little digitonin resulted in an A preparation with some outer membrane still present, whereas too high a ratio led to losses in phosphorylation (see also Schnaitman *et al.* 1967). NADH-cytochrome *c* reductase (rotenone- or antimycin A-insensitive) assays showed that preparation A contained $5.8 \pm 2.9\%$ (22 experiments) of the total activity in mitochondria. The remainder was recovered in the supernatant fraction and washes (crude outer-membrane preparation) of preparation A (Table 1).

Properties of preparation A. Preparation A contained $51 \pm 8\%$ of the original mitochondrial protein (24 experiments). Since the outer membrane probably constitutes approx. 10% of the

total mitochondrial protein (Sottocasa *et al.* 1967) the low recovery of protein in preparation A indicates that some of the inner-membrane complexes are probably also being disrupted or that matrix protein is being lost, or that both are occurring. The possible loss of matrix protein was determined by measuring the release of several enzymes believed to be localized there and readily solubilized (Parsons *et al.* 1967). It should be pointed out, however, that there is no direct evidence that such enzymes exist in a soluble form within the mitochondrion and they may be released because they are easily dislodged from some intra-mitochondrial structure. The results are shown in Table 2 and indicate that in this experiment 70–85% of these enzymes were retained during this first digitonin treatment. The enzyme recoveries from preparation A and the remaining material (crude outer-membrane preparation) was approx. 100%. The results obtained for malate dehydrogenase in eight experiments give an indication of the variability encountered. These were as follows: mitochondrial specific activity, 2.70 ± 0.72 ; recovery in crude outer-membrane preparation, $31 \pm 12\%$; recovery in preparation A, $69 \pm 8\%$; total recovery, $99 \pm 8\%$.

Measurements were also made of the activities of several enzymes believed to be localized in the inner

Table 2. *Effect of digitonin treatment on distribution of 'soluble' mitochondria enzymes*

COM, Crude outer-membrane preparation.

Enzyme	Mitochondria		Recovery (% of total units)			
	Sp. activity (μ mole/min./mg. of protein)	Total units				
			COM	Prep. A	Prep. B	Prep. C
Malate dehydrogenase	2.31	6420	33	72	5.5	3.4
Fumarase	0.87	2418	21	74	4.9	0.9
Glutamate dehydrogenase	0.067	186	15	84	9.3	0.9
Isocitrate dehydrogenase	0.038	105	31	85	3.0	0.8
Protein recovery (%)		100	46	52	9	8

Table 3. *Effect of digitonin treatment on distribution of bound mitochondrial enzymes*

COM, Crude outer-membrane preparation.

Enzyme	Mitochondria		Recovery (% of total units)			
	Sp. activity (μ mole/min./mg. or protein)	Total units				
			COM	Prep. A	Prep. B	Prep. C
NADH-cytochrome <i>c</i> reductase (rotenone-sensitive)	0.059	164	20	82	50	25
Succinate dehydrogenase	0.037	102	16	84	24	10
β -Hydroxybutyrate dehydrogenase	0.505	1396	17	62	17	19
Protein recovery (%)		100	46	52	9	8

mitochondrial membrane (Table 3). In this case about 80% of these enzymes were also retained at this step. The recoveries of enzyme activity were close to 100% except for 3-hydroxybutyrate dehydrogenase. The recovery of the latter varied in the range 69–90% in four other experiments. This lower recovery may possibly result from a phospholipid requirement for solubilized 3-hydroxybutyrate dehydrogenase (Jurtshuk, Sekuzu & Green, 1963). All of the results shown in Tables 1–3 were obtained with fractions obtained from a single mitochondrial preparation. The cytochromes are also believed to be localized in the inner membrane and therefore their distribution should be similar to those enzymes shown in Table 3. In contrast, however, larger losses occurred at this stage (Table 4). The concentrations obtained for whole mitochondria were somewhat lower than those reported by Williams (1964), but the agreement is reasonable. It is assumed that this method is applicable to all of the preparations described.

The P/O ratio of preparation A was usually slightly less than that of the starting mitochondria (Table 5). The specific activity for P_i uptake with 3-hydroxybutyrate or succinate decreased in preparation A but usually was about 45% of that of the mitochondria for 3-hydroxybutyrate and about 20% for succinate. The loss in the ability to

oxidize 3-hydroxybutyrate may, at least in part, be caused by a loss of bound NAD^+ since the addition of NAD^+ markedly stimulates respiration (C. Hoppel & C. Cooper, unpublished work). The increase in specific activity for P_i uptake with cytochrome *c* plus ascorbate as substrate may have resulted from the removal of a permeability barrier as a result of the conversion of mitochondria into the other preparations or an enrichment of the enzymes involved. The change in the P_i -ATP exchange activity on conversion of mitochondria into preparation A usually paralleled that of the P_i uptake with 3-hydroxybutyrate as substrate. Both the oxidative phosphorylation and the P_i -ATP exchange were completely inhibited by 1.5 μ g. of oligomycin/ml. in all preparations. The Mg^{2+} -stimulated adenosine triphosphatase increased in specific activity by 144% in preparation A and represented 75% of the total activity of the starting mitochondria. The remainder was completely recovered in the crude outer-membrane fraction.

Properties of preparations B and C. Table 1 shows that the microsomal enzyme NADPH-cytochrome *c* reductase (rotenone-insensitive) was not detectable in preparation C. The values obtained for this assay with the other preparations may be somewhat high, since no correction was applied for possible changes in the light-scattering properties of the

Table 4. *Effect of digitonin treatment on distribution of cytochromes*

Results are given as means $\pm$ s.d., with the numbers of experiments in parentheses. COM, Crude outer-membrane preparation.

Cytochrome	Concn. of mitochondria (m μ mole/mg. or protein)	Recovery (% of total)			
		COM	Prep. A	Prep. B	Prep. C
Cytochrome <i>b</i>	0.20 $\pm$ 0.05 (5)	65 $\pm$ 3 (4)	44 $\pm$ 14 (5)	39 $\pm$ 7 (3)	28 (1)
Cytochrome <i>c</i> ₁	0.11 $\pm$ 0.01 (5)	65 $\pm$ 7 (4)	29 $\pm$ 13 (5)	24 $\pm$ 7 (3)	18 (1)
Cytochrome <i>c</i>	0.25 $\pm$ 0.01 (5)	63 $\pm$ 11 (4)	41 $\pm$ 12 (5)	33 $\pm$ 8 (3)	25 (1)
Cytochrome <i>a</i> + <i>a</i> ₃	0.21 $\pm$ 0.04 (5)	43 $\pm$ 18 (4)	61 $\pm$ 18 (5)	48 $\pm$ 5 (3)	38 (1)
Protein recovery (%)	100	49 $\pm$ 6 (24)	51 $\pm$ 8 (24)	13 $\pm$ 4 (18)	8 $\pm$ 1 (4)

Table 5. *Effect of digitonin treatment on enzyme systems related to oxidative phosphorylation*

Results for rows 2–7 are specific activities (m μ moles/min./mg. of protein). The numbers of experiments are given in parentheses.

Quantity measured	Mitochondria	Prep. A	Prep. B	Prep. C
3-Hydroxybutyrate $\rightarrow$ O ₂ (P/O)	2.5 $\pm$ 0.2 (18)	2.0 $\pm$ 0.2 (18)	1.5 $\pm$ 0.2 (18)	1.3 $\pm$ 0.1 (4)
3-Hydroxybutyrate $\rightarrow$ O ₂ (P_i uptake)	63 $\pm$ 11 (18)	28 $\pm$ 10 (8)	25 $\pm$ 11 (18)	21 $\pm$ 9 (4)
3-Hydroxybutyrate $\rightarrow$ O ₂ (acetoacetate formation)	24 $\pm$ 2 (18)	14 $\pm$ 5 (18)	17 $\pm$ 7 (18)	17 $\pm$ 8 (4)
Cytochrome <i>c</i> + ascorbate $\rightarrow$ O ₂ (P_i uptake)	9 $\pm$ 1 (18)	13 $\pm$ 5 (18)	29 $\pm$ 16 (18)	28 $\pm$ 7 (4)
Succinate $\rightarrow$ O ₂ (P_i uptake)	107 $\pm$ 13 (5)	23 $\pm$ 16 (4)	31 $\pm$ 17 (4)	24 (1)
P_i -ATP exchange	68 $\pm$ 11 (18)	36 $\pm$ 10 (18)	14 $\pm$ 7 (18)	11 $\pm$ 5 (4)
Adenosine triphosphatase	160 (1)	230 (1)	485 (1)	495 (1)
Protein recovery (%)	100	51 $\pm$ 8 (24)	13 $\pm$ 4 (18)	8 $\pm$ 1 (4)

protein during the assay (Phillips & Langdon, 1962). NADH-cytochrome *c* reductase (rotenone-insensitive), an outer-membrane enzyme, was also not detectable in preparation C. There was further enrichment of the NADH-cytochrome *c* reductase (rotenone-sensitive) in the C preparation, the specific activity being 312% of that observed in the starting mitochondria (Table 3). Succinate dehydrogenase and 3-hydroxybutyrate dehydrogenase were also enriched and had specific activities of 125% and 236% respectively that of the starting mitochondria. These enzymes were solubilized at this step and their enrichment was not nearly as great as seen with the NADH-cytochrome *c* reductase (rotenone-sensitive) (Table 3). The cytochromes were largely retained in the particulate fractions after the initial losses. The C preparation was considerably enriched in cytochromes compared with the mitochondria: 475% for *a*+*a*₃, 314% for *c*, 263% for *c*₁ and 340% for *b* (Table 4). Adenosine triphosphatase was also enriched and had a specific activity 309% of that of the mitochondria (Table 5).

The enzymes shown in Table 2 represent those released when the inner mitochondrial unit was disrupted. Small amounts of malate dehydrogenase were usually present in preparation C, whereas the others were absent or barely detectable.

Preparation C carried out phosphorylation coupled to oxidation of 3-hydroxybutyrate, cytochrome *c* + ascorbate or succinate, and P_i-ATP exchange (Table 5). The low recovery of exchange activity relative to the P_i uptake with different substrates may result from the fact that the assay for the exchange is dependent on the bound Mg²⁺ whereas the assays for P_i uptake are made with added Mg²⁺. None of the changes produced in going from preparation B to preparation C could be reproduced by one to five washes of preparation B with water.

Effect of water and digitonin on preparation A. The properties of the A preparation were markedly altered when it was suspended in water instead of 0.3M-sucrose. This may be seen in Table 6 by contrasting the columns labelled 'A' and 'A₁'. Suspension in water (preparation A₁) led to a complete loss of P_i-ATP exchange and of phosphorylation with 3-hydroxybutyrate as substrate and a very large inhibition of phosphorylation when succinate or cytochrome *c* + ascorbate were the substrates. This does not appear to have been caused by a complete loss in oxidase activity since the specific activity for acetoacetate formation was still 33% of that of preparation A. When the water suspension (preparation A₁) was sedimented at 45000g almost all of the protein and malate dehydrogenase and 3-hydroxybutyrate dehydrogenase activities were recovered in the pellet and

there was some enhancement of phosphorylation and P_i-ATP exchange (preparation A₂; Table 6). At this protein concentration of the A₁ suspension (130mg./ml.) the properties of preparation A₂ were not significantly altered by repeating the procedure or by prolonging the incubation in water before centrifugation. However, if the protein concentration was decreased during the water treatment to 75mg. of protein/ml., only 75% of the protein was recovered (rather than 92%) in the A₂ pellet. When the A₂ preparation was treated with digitonin there was a loss of protein and dehydrogenases but a very marked increase in phosphorylation and exchange activities and a substantial increase in the P/O ratio (preparation B₃; Table 6). It should be noted that this increase cannot be accounted for in the case of the P_i uptake with 3-hydroxybutyrate and the P_i-ATP exchange by assuming that the enhanced specific activity merely resulted from the loss of inactive protein.

If the A preparation was suspended in water for 30min. (preparation A₁) and digitonin added before centrifugation the pellet obtained had a good P/O ratio and the other properties were also different from those of preparations A₁ and A₂ (preparation B₄; Table 6). A prolonged incubation with digitonin was not necessary since preparation A could be suspended directly in aqueous digitonin and centrifuged immediately and the pellet had properties very similar to those of the other B preparations (preparation B₅; Table 6). Other experiments (not shown) indicated that the phosphorylation activities of preparation B₅ were consistently greater than those of preparations B₃ and B₄ if comparable concentrations of digitonin were employed or if the preparations were washed before being assayed.

Effect of suspending-buffer concentration on preparation A. Table 7 shows that preparation A responded to changes in buffer concentration. Relatively high osmoticity gave much greater P/O ratios and enzyme activities. In these experiments a concentrated suspension of preparation A containing 90mg. of protein/ml. of 0.01M-*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffer was diluted to 3.6mg./ml. with the various solutions noted, kept at 0° for 20min. and then assayed. The greatest activity was seen when the preparation was diluted in 0.1M buffer and the lowest when diluted with water or 1mM buffer.

Stability on storage. A major problem in making these preparations has been the lengthy procedure involved and our inability to maintain certain activities on storage. Grieff & Myers (1961) reported that they were able to maintain the activity of rat liver mitochondria on prolonged storage in 10% dimethyl sulphoxide. We have been able to freeze and store mitochondria for long

Table 6. *Effect of treatment of preparation A on properties of preparation B*

Results for rows 2-6 are specific activities ($\mu\text{moles/min./mg. of protein}$). Preparation A₁ was prepared by resuspending a pellet of preparation A in water (130 mg. of protein/ml.); the portion used for the assay was diluted to 3.6 mg. of protein/ml. with water. Preparation A₂ was prepared by keeping preparation A₁ at 0° for 30 min., diluting with 3 vol. of water and then centrifuging for 20 min. at 45000g; the pellet was resuspended in water (3.6 mg. of protein/ml.). Preparation B₃ was prepared as described for preparation A₂ except that the pellet was resuspended in 1% digitonin (1.5 mg. of digitonin/10 mg. of protein), kept for 20 min. at 0°, diluted with 3 vol. of water and then centrifuged for 30 min. at 100000g (Spinco model L, no. 40 rotor); the pellet was resuspended in water (3.6 mg. of protein/ml.). Preparation B₄ was prepared by keeping preparation A₁ at 0° for 30 min., then adding digitonin (1.4 mg. of digitonin/10 mg. of protein), keeping it for 20 min. at 0°, diluting with 3 vol. of water and then centrifuging for 30 min. at 100000g; the pellet was resuspended in water (3.6 mg. of protein/ml.). Preparation B₅ was prepared by resuspending a pellet of preparation A in 1% digitonin (2.9 mg. of digitonin/10 mg. of protein) and immediately centrifuging for 30 min. at 100000g; the pellet was resuspended in water (3.6 mg. of protein/ml.).

Quantity measured	Mitochondria	Prep. A	Prep. A ₁	Prep. A ₂	Prep. B ₃	Prep. B ₄	Prep. B ₅
β -Hydroxybutyrate $\rightarrow$ O ₂ (P/O)	2.3	2.5	0	0.8	1.6	1.5	1.7
β -Hydroxybutyrate $\rightarrow$ O ₂ (P ₁ uptake)	64	30	0	5	33	15	26
β -Hydroxybutyrate $\rightarrow$ O ₂ (acetoacetate formation)	28	12	4	6	21	10	15
Cytochrome c + ascorbate $\rightarrow$ O ₂ (P ₁ uptake)	5	8	2	10	36	20	27
Succinate $\rightarrow$ O ₂ (P ₁ uptake)	90	7	3	14	12	20	10
P ₁ -ATP exchange	81	45	0	3	27	6	12
Malate dehydrogenase (% recovery)	100	59	59	56	9	15	7
3-Hydroxybutyrate dehydrogenase (% recovery)	100	62	62	58	30	36	13
Protein recovery (%)	100	49	49	45	12	18	9

Table 7. *Effect of buffer concentration on some activities of preparation A*

Results for rows 2 and 3 are specific activities ($\mu\text{moles/min./mg. of protein}$). TES, *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid.

Prep. A (90 mg./ml. in 0.01 M-TES, pH 6.8) diluted to 3.6 mg./ml.

Reaction	Prep. A (90 mg./ml. in 0.01 M-TES, pH 6.8) diluted to 3.6 mg./ml.			
	With water	With 1 mM-TES, pH 6.8	With 0.01 M-TES, pH 6.8	With 0.1 M-TES, pH 6.8
3-Hydroxybutyrate $\rightarrow$ O ₂ (P/O)	1.2	1.1	1.8	2.9
3-Hydroxybutyrate $\rightarrow$ O ₂ (P ₁ uptake)	4	4.7	6.7	17
P ₁ -ATP exchange	3.2	2.8	3.2	10

periods with only moderate loss of activity after 82 days and B preparations for 6 months without loss of phosphorylation (Table 8). The protein concentration was important (Table 8) and the optimum was 30-40 mg. of protein/ml. of 10% dimethyl sulphoxide. At 100 mg./ml. there was a loss of approx. 50%. The dimethyl sulphoxide purchased commercially has a yellowish colour that can be removed by vacuum distillation, but the distilled dimethyl sulphoxide was not nearly as effective a protective agent.

DISCUSSION

Though digitonin has received wide use for the solubilization of lipoproteins, little is known about

how it acts. It has been suggested that it may combine with free cholesterol to form a digitonide and that this in turn leads to 'solubilization' or disruption (Schnaitman *et al.* 1967). Siekevitz & Watson (1957) found that digitonin appears to have an effect on rat liver mitochondria different from that of deoxycholate. When a pellet of mitochondria was overlaid with deoxycholate and sections were made from various points within the pellet it was apparent that the mitochondria were swollen and disrupted in proportion to the amount of deoxycholate that had penetrated. In contrast, digitonin appeared to produce no intermediate stages between intact mitochondria and rather small vesicular units. Levy *et al.* (1966) and Schnaitman *et al.* (1967) have provided electron micrographs

Table 8. *Stability on storage*

		DMSO, Dimethyl sulphoxide.		
		Sp. activity (μ moles/min./mg. of protein)		
Preparation and storage conditions	P/O ratio	3-Hydroxy- butyrate $\rightarrow$ O ₂ (P ₁ uptake)	Cytochrome c + ascorbate $\rightarrow$ O ₂ (P ₁ uptake)	P ₁ -ATP exchange
Mitochondria				
As prepared	2.5	65	10	57
Frozen for 21 days in DMSO	2.7	63	12	48
Frozen for 82 days in DMSO	2.4	44	15	46
Preparation B				
As prepared	1.3	10	6	4
Frozen for 1 day without DMSO	0	0	0	0
Frozen for 7 days in DMSO	1.9	13	12	4
Frozen for 181 days in DMSO	1.1	13	13	5
Mitochondria				
As prepared	2.3	65	8	62
Suspension containing 32 mg. of protein/ml. frozen for 1 day in undistilled DMSO	2.3	61	8	59
Suspension containing 32 mg. of protein/ml. frozen for 1 day in distilled DMSO	2.2	48	9	46
Suspension containing 110 mg. of protein/ml. frozen for 1 day in undistilled DMSO	2.3	34	6	33

demonstrating that when mitochondria suspended in 0.25M-sucrose were treated with digitonin the outer membrane was removed and converted into small empty vesicles that could be isolated at 105 000g. In addition, the latter group reported the isolation of an intermediate fraction at 40 000g consisting of swollen and partially disrupted inner membranes. We have confirmed these findings with regard to the removal of outer membrane by digitonin treatment. This was demonstrated by the removal of 95% of the NADH-cytochrome *c* reductase (rotenone-insensitive) (Table 1) whereas the resulting structure still retained 70–85% of the 'soluble' mitochondrial enzymes (Table 2) and the bound mitochondrial enzymes (Table 3). These findings are supported by electron micrographs (Morton, Hoppel & Cooper, 1968). The relatively high loss of cytochromes (Table 4) at this point was unexpected, but the finding is reproducible and may result from a preferential solubilization of cytochromes from the structure also containing succinate dehydrogenase etc. or from differences in the types of structures to which they are bound. It may be noted that, whereas the NADH-cytochrome *c* reductase and succinate dehydrogenase were both appreciably lost during the conversion of preparation B into preparation C, there was no loss of 3-hydroxybutyrate dehydrogenase (Table 3).

Though the effect of digitonin on intact mitochondria appears to result in a selective removal of the outer membrane it is apparent that a further treatment of the resulting preparation A resulted

in a disruption of the inner membrane as well. This is confirmed by electron-microscopic examination (Morton *et al.* 1968) and appears to be caused by a specific effect of digitonin on the inner mitochondrial complex rather than the hypo-osmotic conditions prevailing during the digitonin treatment.

If preparation A was treated with water it underwent swelling as evidenced by the increase in pellet volume, and the resulting particulate fraction, A₂, contained most of the protein of preparation A but showed low phosphorylation and exchange activity. If this preparation was centrifuged and the isolated pellet then treated with digitonin its activity increased markedly and the protein recovery decreased to the level that would have been obtained had preparation A been treated directly with digitonin. Thus the water treatment produced a loss of activity that was reversed by digitonin treatment. The reason for the increase in phosphorylation and exchange activity is obscure, but it is not caused by a removal of inactive protein. It should also be noted that it does not require the addition of any mitochondrial proteins. It may arise as a result of the removal of some inhibitory material or perhaps a reorientation of an enzymically inactive lipoprotein complex.

The location of the phosphorylation enzymes in the mitochondrion has still not been firmly established. The relatively low content of succinoxidase, cytochrome oxidase and the α -type cytochromes in the outer membrane (Sottocasa *et al.* 1967; Parsons *et al.* 1967) indicates that the

respiratory chain and therefore oxidative phosphorylation is associated with the inner portion of the mitochondrion and not with the outer membrane. The consensus at present is that these enzymes are bound to the inner membrane, but until pure inner membrane free of other material is isolated and tested the question must remain open. Our crude outer-membrane fraction carried out coupled oxidative phosphorylation probably because it was contaminated with material originating from the inner mitochondrial complex. The particulate fraction isolated after centrifugation of the crude outer-membrane fraction at 100000g most closely resembles the digitonin preparation originally described (Cooper & Lehninger, 1956). This is supported by the finding that preparations made as described by Cooper & Kulka (1961) and Kulka & Cooper (1962) had a specific activity of 1.1–1.2 μ moles/min./mg. of protein for the outer-membrane enzyme NADH–cytochrome c reductase (rotenone-insensitive). This indicates a considerable enrichment over the activity of intact mitochondria (0.27 ± 0.08 in 24 experiments) and contrasts sharply with preparation C, which was devoid of this enzyme.

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